

24098-168

Appelbaum/Dad 9/12/95

# EXPRESSION OF TNF-LIKE PROTEIN

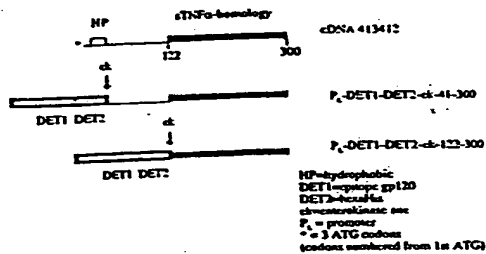
HGS clone 413412 = HTPAN98

## Objectives:

- Express as fusion protein in E. coli—to be used for raising antibodies
- Express in soluble form in E. coli or other systems—to be used for receptor binding and activity assays

## Accomplishments:

- Plasmid DNA from HGS strain (purified by K. B. Tan) was sequenced.
- The DNA sequence in the open reading frame agreed with that reported by HGS.
- There was a 1 bp difference upstream of the first of 3 closely spaced in-frame ATG codons, which makes it more likely that the first ATG is the translation initiator codon (TTCAATGG in HGS sequence is ATCAATGG in SB sequence; the latter is in good agreement with Kozak consensus upstream sequence)
- Two fusion constructs being made for E. coli expression; both have the potential for release of soluble protein following enterokinase digestion.



IDENTITY of: Tnf\_ human check: 6906 from: 1 to: 233

ID Tnf\_ human STANDARD: PRT: 233 AA.

AC P01773

DT 21-JUN-1994 INCL. 01. CREATION

DT 21-JUN-1994 INCL. 02. LAST SEQUENCE UPDATE

DT 01-JUN-1994 INCL. 29. LAST AMINO ACID UPDATE

DE Tumor necrosis factor precursor (TNF-ALPHA) (CONJECTURE)

on 413412.Pap check: 2906 from: 1 to: 279

TRANSLATE of: 413412 check: 8776 from: 54 to: 1443

generated symbols: 1 to: 528

NAME 413412

DEFINITION P10 ligand (non-membrane)

ACCESSION U03412

KEYWORDS TNF, human, precursor, tumor

Symbol comparison table: Conserved: 1 (Gpcore, Data, Rndact) Dgppppp, Cdp

CompCheck: 1234

Gap length: 7.000 Average match: 0.500

Length: 0.250 Average mismatch: -0.250

Quality: 75.5 Length: 233

Ratio: 0.732 Gaps: 2

Percent similarity: 33.333 Percent identity: 24.814

Tnf\_ human & 413412.Pap July 11, 1995 15:06

1 5 QHSDVLAAGALPHYOCPOCNCLFLSLFSLVACATLPCLLAWC 54

10 10 10 10 10 10 10 10 10 10 10 10 10 10 10 10

15 15 15 15 15 15 15 15 15 15 15 15 15 15 15 15

20 20 20 20 20 20 20 20 20 20 20 20 20 20 20 20

25 25 25 25 25 25 25 25 25 25 25 25 25 25 25 25

30 30 30 30 30 30 30 30 30 30 30 30 30 30 30 30

35 35 35 35 35 35 35 35 35 35 35 35 35 35 35 35

40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40

45 45 45 45 45 45 45 45 45 45 45 45 45 45 45 45

50 50 50 50 50 50 50 50 50 50 50 50 50 50 50 50

55 55 55 55 55 55 55 55 55 55 55 55 55 55 55 55

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100 100 100 100 100 100 100 100 100 100 100 100 100 100 100

105 105 105 105 105 105 105 105 105 105 105 105 105 105 105

110 110 110 110 110 110 110 110 110 110 110 110 110 110 110

115 115 115 115 115 115 115 115 115 115 115 115 115 115 115

120 120 120 120 120 120 120 120 120 120 120 120 120 120 120

125 125 125 125 125 125 125 125 125 125 125 125 125 125 125

130 130 130 130 130 130 130 130 130 130 130 130 130 130 130

135 135 135 135 135 135 135 135 135 135 135 135 135 135 135

SCIENTIST SIGNATURE

*[Handwritten signature]*

*[Handwritten signature]*

Ruben EXHIBIT #32

BEST AVAILABLE COPY



No. 24098-168

Appelbaum/Dal 9/12/95

# EXPRESSION OF TNF-LIKE PROTEIN

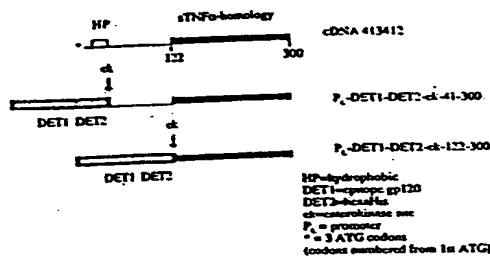
HGS clone 413412 = HTFAN08

## Objectives:

- Express as fusion protein in E. coli—to be used for raising antibodies
- Express in soluble form in E. coli or other systems—to be used for receptor binding and activity assays

## Accomplishments:

- Plasmid DNA from HGS strain (purified by K. B. Tan) was sequenced.
- The DNA sequence in the open reading frame agreed with that reported by HGS.
- There was a 1 bp difference upstream of the first of 3 closely spaced in-frame ATG codons, which makes it more likely that the first ATG is the translation initiator codon (TTCAATGG in HGS sequence is ATCAATGG in S8 sequence; the latter is in good agreement with Kozak consensus upstream sequence)
- Two fusion constructs being made for E. coli expression; both have the potential for release of soluble protein following enterokinase digestion.



SEQUENCE of: TNFα\_human check: 6906 from: 1 to: 233

ID: TNFα\_human STANDARD: PRT: 233 AA.

AC: P01375

DT: 21-JUL-1994 (REL. 01. CREATED)

DT: 21-JUL-1994 (REL. 01. LAST SEQUENCE UPDATE)

DT: 01-JUN-1994 (REL. 29. LAST AMOUNT/DATE UPDATE)

DE: TUMOR NECROSIS FACTOR PRODUCTION (TNF-ALPHA) (CHEMOKINE) . . . .

CO: 413412.Pap check: 5940 from: 1 to: 279

TRANSLATE of: 413412 check: 8736 from: 50 to: 1643

Generated symbols: 1 to: 530.

LOCUS: 413412

DEFINITION: Pox ligand (Shu protein)

ACCESSION: 523412

LIBRARY: HTFAN08 Plasmid Tumor . . . .

Symbol comparisons table: GeneCrawler: (CpGeneData.BioData)SeqComp.Cmp

CmpCheck: 1254

Cap Weight: 7.000 Average Match: 0.340

Length Weight: 0.250 Average Mismatch: -0.294

Quality: 75.0 Length: 233

Ratio: 0.212 Gaps: 2

Percent Similarity: 33.394 Percent Identity: 16.414

TNFα\_human = 413412.Pap July 11, 1995 15:46 . .

5 SHIRVCEALALPRTYQPCENACLFSLFSLTVCATYLLPCLNG 54

60 SCLACFLKEDDTYQPCENACLFSLFSLTVCATYLLPCLNG 99

65 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 103

70 ISTVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 147

75 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 149

80 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 197

85 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 199

90 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 243

95 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 245

100 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 272

105 VIGVQRECFVDELITSLPQVPELITSLPQVPELITSLPQV 278

SCIENTIST SIGNATURE

*Edward J. Appelbaum*

DATE

*11/12/95*

*Partner James A. Donald*

*Feb 9 1996*

SITE OF START  
DET1-DET2 EK  
122-300

## SYNTHETIC DNA REQUEST FORM

Please read these instructions before you write your oligos:

1. Please write all oligo sequences from 5' to 3' direction.
2. Write sequences in capital letters using a pen (Not pencil).
3. You can request more than one oligo on a same page.
4. Write your name, project, department and phone number.
5. Write your sequences as 5' ATG - CCC - GAA - TTC - AGA - TTT 3'
6. If you need specially oligos (Biotin, Amino, Uracil, Dmso, etc.), please write them accordingly.

## OLIGO SEQUENCES:

1. 5' CGG - GAT - CCT - CGA - CGA - CGA - CAA - ACA - GCT -  
GAA - GCA - GAT - GCA - GGA 3' SB 6686
2. 5' CCG - GAT - CCT - CGA - CGA - CGA - CCA - AAT - AGC - AGC -  
TCA - CAT - AAT - TGG 3' SB 6687
3. ~~5' CCG - GAT - CCT - CGA - CGA - CGA - CCA - AAT - AGC - AGC -  
TCA - CAT - AAT - TGG 3' SB 6688~~
4. 5' GCT - CTA - CAT - TAG - CCA - ALT - AAA - AAG - GLL -  
GAA - GCA - GAT - GCA - GGA 3' SB 6689

Requestor: EDWARD DUL

Project: CHIMES

Department: GCS

Telephone Number: 7788

Indicate for what purpose you are requesting these oligos:

☒ PCR primers      Sequencing primers      Linkers      Probes  
☐ Antisense      Gene construction      Affinity columns

Hematology Clinical Section, Los Tujos or Joyce Shaw, Ext 3689 for help.

Please to: Mr. Ted Lee 475-545 - R 100.

These oligos were designed  
 from the DNA sequence of  
 HGS clone 413412 (ATG-343)  
 provided by HGS

HGS clone 413412 (ATG 343) was sequenced in its  
 entirety and was in agreement with the HGS sequence.  
 This was the template for the PCR reaction of the TNF-like  
 protein.

L1, L2 designate PCR reactions to obtain  
 DRT1, DRT2 & K 41-300

S1, S2 designate PCR reactions to obtain DRT1, DRT2  
 & K' 122-300

SCIENTIST SIGNATURE

Edward Dul

DATE 11/17/95

written by James O'Neill Feb 7, 1996

24098-170

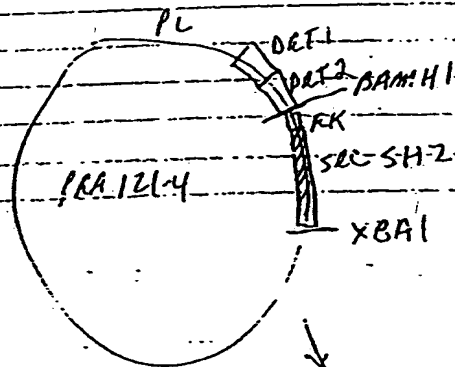
ATC 343-2-1/1

1.2500/500 H<sub>2</sub>O

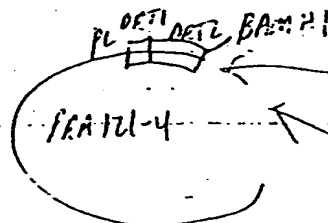
	ATC 343	ATC 343	ATC 343	ATC 343
	1.25	1.25	1.25	1.25
SB 6696 (44-100)	1.38	95	1.38	107
SB 6697 (45-100)	1.13	107	1.13	157
SB 6698 (27-100)	1.40	157	1.40	
ATC 343 (100-1)	1.25	1.25	1.25	1.25
SB 6696	1.25	1.25	1.25	1.25
SB 6697	1.25	1.25	1.25	1.25
SB 6698	1.25	1.25	1.25	1.25
SB 6699	1.25	1.25	1.25	1.25
SB 6700	1.25	1.25	1.25	1.25
SB 6701	1.25	1.25	1.25	1.25
SB 6702	1.25	1.25	1.25	1.25
SB 6703	1.25	1.25	1.25	1.25
SB 6704	1.25	1.25	1.25	1.25
SB 6705	1.25	1.25	1.25	1.25
SB 6706	1.25	1.25	1.25	1.25
SB 6707	1.25	1.25	1.25	1.25
SB 6708	1.25	1.25	1.25	1.25
SB 6709	1.25	1.25	1.25	1.25
SB 6710	1.25	1.25	1.25	1.25
SB 6711	1.25	1.25	1.25	1.25
SB 6712	1.25	1.25	1.25	1.25
SB 6713	1.25	1.25	1.25	1.25
SB 6714	1.25	1.25	1.25	1.25
SB 6715	1.25	1.25	1.25	1.25
SB 6716	1.25	1.25	1.25	1.25
SB 6717	1.25	1.25	1.25	1.25
SB 6718	1.25	1.25	1.25	1.25
SB 6719	1.25	1.25	1.25	1.25
SB 6720	1.25	1.25	1.25	1.25
SB 6721	1.25	1.25	1.25	1.25
SB 6722	1.25	1.25	1.25	1.25
SB 6723	1.25	1.25	1.25	1.25
SB 6724	1.25	1.25	1.25	1.25
SB 6725	1.25	1.25	1.25	1.25
SB 6726	1.25	1.25	1.25	1.25
SB 6727	1.25	1.25	1.25	1.25
SB 6728	1.25	1.25	1.25	1.25
SB 6729	1.25	1.25	1.25	1.25
SB 6730	1.25	1.25	1.25	1.25
SB 6731	1.25	1.25	1.25	1.25
SB 6732	1.25	1.25	1.25	1.25
SB 6733	1.25	1.25	1.25	1.25
SB 6734	1.25	1.25	1.25	1.25
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SB 6736	1.25	1.25	1.25	1.25
SB 6737	1.25	1.25	1.25	1.25
SB 6738	1.25	1.25	1.25	1.25
SB 6739	1.25	1.25	1.25	1.25
SB 6740	1.25	1.25	1.25	1.25
SB 6741	1.25	1.25	1.25	1.25
SB 6742	1.25	1.25	1.25	1.25
SB 6743	1.25	1.25	1.25	1.25
SB 6744	1.25	1.25	1.25	1.25
SB 6745	1.25	1.25	1.25	1.25
SB 6746	1.25	1.25	1.25	1.25
SB 6747	1.25	1.25	1.25	1.25
SB 6748	1.25	1.25	1.25	1.25
SB 6749	1.25	1.25	1.25	1.25
SB 6750	1.25	1.25	1.25	1.25
SB 6751	1.25	1.25	1.25	1.25
SB 6752	1.25	1.25	1.25	1.25
SB 6753	1.25	1.25	1.25	1.25
SB 6754	1.25	1.25	1.25	1.25
SB 6755	1.25	1.25	1.25	1.25
SB 6756	1.25	1.25	1.25	1.25
SB 6757	1.25	1.25	1.25	1.25
SB 6758	1.25	1.25	1.25	1.25
SB 6759	1.25	1.25	1.25	1.25
SB 6760	1.25	1.25	1.25	1.25
SB 6761	1.25	1.25	1.25	1.25
SB 6762	1.25	1.25	1.25	1.25
SB 6763	1.25	1.25	1.25	1.25
SB 6764	1.25	1.25	1.25	1.25
SB 6765	1.25	1.25	1.25	1.25
SB 6766	1.25	1.25	1.25	1.25
SB 6767	1.25	1.25	1.25	1.25
SB 6768	1.25	1.25	1.25	1.25
SB 6769	1.25	1.25	1.25	1.25
SB 6770	1.25	1.25	1.25	1.25
SB 6771	1.25	1.25	1.25	1.25
SB 6772	1.25	1.25	1.25	1.25
SB 6773	1.25	1.25	1.25	1.25
SB 6774	1.25	1.25	1.25	1.25
SB 6775	1.25	1.25	1.25	1.25
SB 6776	1.25	1.25	1.25	1.25
SB 6777	1.25	1.25	1.25	1.25
SB 6778	1.25	1.25	1.25	1.25
SB 6779	1.25	1.25	1.25	1.25
SB 6780	1.25	1.25	1.25	1.25
SB 6781	1.25	1.25	1.25	1.25
SB 6782	1.25	1.25	1.25	1.25
SB 6783	1.25	1.25	1.25	1.25
SB 6784	1.25	1.25	1.25	1.25
SB 6785	1.25	1.25	1.25	1.25
SB 6786	1.25	1.25	1.25	1.25
SB 6787	1.25	1.25	1.25	1.25
SB 6788	1.25	1.25	1.25	1.25
SB 6789	1.25	1.25	1.25	1.25
SB 6790	1.25	1.25	1.25	1.25
SB 6791	1.25	1.25	1.25	1.25
SB 6792	1.25	1.25	1.25	1.25
SB 6793	1.25	1.25	1.25	1.25
SB 6794	1.25	1.25	1.25	1.25
SB 6795	1.25	1.25	1.25	1.25
SB 6796	1.25	1.25	1.25	1.25
SB 6797	1.25	1.25	1.25	1.25
SB 6798	1.25	1.25	1.25	1.25
SB 6799	1.25	1.25	1.25	1.25
SB 6800	1.25	1.25	1.25	1.25

cut clone for fragments with BamHI/XBAI

Digest pRA121-4 with BAMHI/XBAI



BAMHI/XBAI



ATC 343  
L1 L2 end  
ATC 343  
S1 S2

cut with BAMHI/XBAI

H<sub>2</sub> (150 BP)

S1 (2500 BP)

ligate

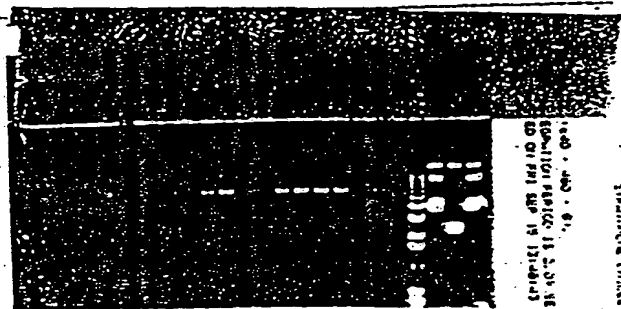
SCIENTIST SIGNATURE

*Richard Hef*

DATE

11/17/95

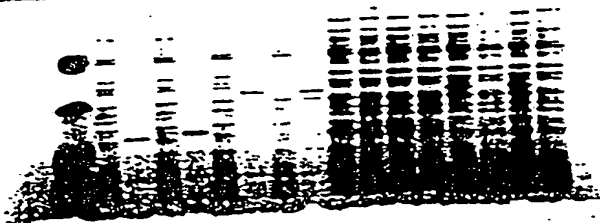
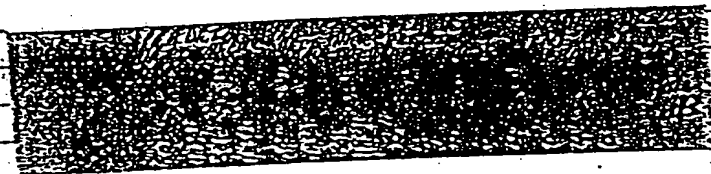
Witness: Peter A. Poulak Feb 9, 1996



Sequence of candidates L1 F3, S2 F3 are as expected

more dense constructs in L1 F4

EXPERIMENTAL DATA TO BE USED



INDUCTIONS (LW14)  
PRA DAT1 DAT2 PER TAF-LIKE

	0'	4'
L-1-3	.586	1.5
L-2-2	.638	1.63
S-1-3	.614	1.67
S-2-3	.606	1.63

normalize pullets to 3D  
including self mass  
fraction

Whole cells  
Coomassie 100  
Western 14

Fractions  
Coomassie 200  
Western 60

SCIENTIST SIGNATURE

*Edward J. Hall*

DATE

11/17/95

Induction from C. F. Hall Feb 9, 1996

24098-172

Wrote  
cells

STORAGE ENCLAVE 10-16-95 14:00:00

IMAGE SIZE (640 x 480 x 8)



Run Western Blot of above sample using 178-1 (D.F.I. antisera)

Band on creamasee lights up using D.F.I. Antisera (178-1)

SCIENTIST SIGNATURE

Richard D. Hall

DATE 11/17/95

return to AT Smith 527, 1996

INF-Like gene (ATG-343) now called TL2-L (long)  
and TL2-S (short)

Chris Jones purified a 1 liter induction of  
TL2-L (Both DNA sequences of TL2 DRT1 DRT2  
forms have been confirmed as expected)

Material was highly insoluble and C. fuel led to  
solubility. It is soluble and dilute it off  
obtained ~ 30 mg material in 65 ml

Inject in rabbits for Antisera

To determine solubility <sup>at low Temp</sup> Transform DRT1 DRT2 & TL2  
(Both forms L and S) in G198 with Tryptophan  
induction at 28° and 32°

		0	4
1) G198	TL2-L1 (28°)	.460	1.19
2) G198	TL2-S2 (28°)	.436	1.07
4) G198	TL2-S2 (32°)	.407	1.16

whole cell pellets / sol/insol fractions

\* Have obtained from HGS 2 Antisera to TL2 (ATG?)  
call HGS TL2-1 and HGS TL2-2

Obtained pre-blend from TL2 rabbits here (Both  
pre-blend and 1st blend - called CK9A and CK9B

I was unable to see expression as well in this  
strain as I was able to see with AR58 & AR170

Run Westerns (crude cells/100ul - sol/insol 30 ul  
100ng DRT1 DRT2 TL2 purified by Chris Jones  
1/1000 dilutions of RACK

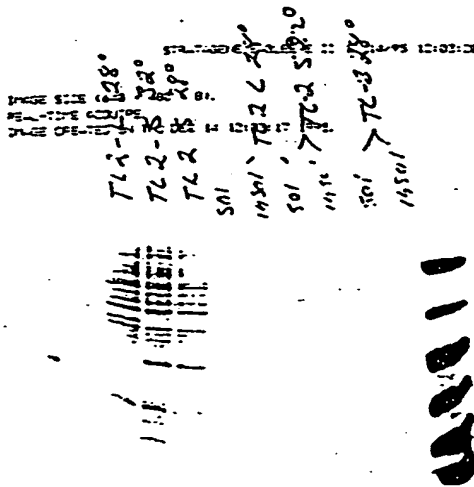
SCIENTIST SIGNATURE

DATE

Refused to sign

Antisera (Jones & Powell) 2/11/96  
Feb 9, 1996

Western Blot showed recognition of purified Tc2 with  
 both myc anti sera (CK9B) but much more faintly let val,  
 with the HCS anti sera Tc2-2



NH2 Terminal of CKB-13 purified from *Phasophila*  
 permits recognition of CKB-13 in cells (PMK)

SCIENTIST SIGNATURE

*Edward [Signature]*

DATE 2/1/96

Written by J. A. Powell Feb 9, 1996

62699 induction of PEA DET1 DET2 TL2

overnight OD<sup>550</sup> checked

L1-28°C - 3.5

S2-28°C - 3.1

L1-32°C - 4.8

S2-32°C - 4.6

Dilute Each ON in Induction media

	vol of n	Vol Ind media	OD <sup>550</sup> Diluted cells
L1-28°	1.5	25 ml	269
S2-28°	2	" "	221
L1-32°	1	" "	258
S2-32°	1	" "	244

1 hr 15 min later

	2'	4' after addition of 100 µg/ml tetracycline
(1) L1-28°	530	197
(2) S2-28°	463	7.0
(3) L1-32°	547	2.26
(4) S2-32°	445	2.14

normalize whole cells in lysis Buffer

set aside cells for sonifications - normalize them in Buffer A + lysozyme

inc - 30 min

sonicate

spin in microfuge - 30 min

SCIENTIST SIGNATURE

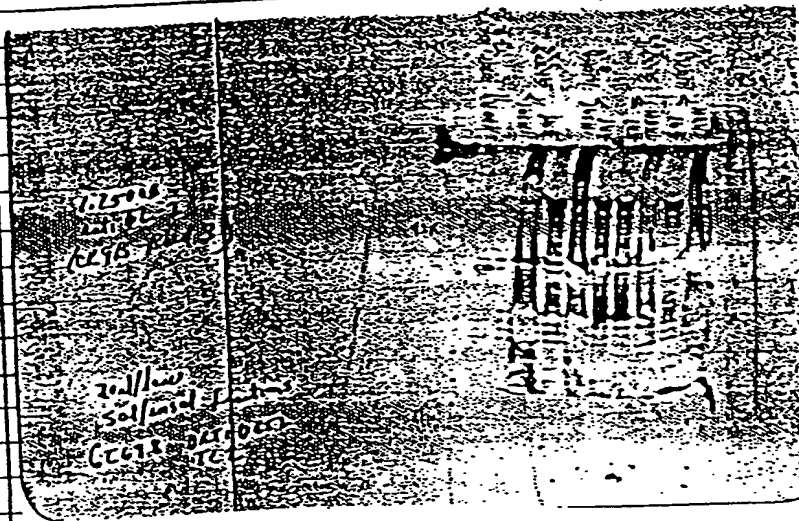
Richard D. Hall

DATE 2/8/96

Inters. J. Am. Acad. Feb 91 1996

Run 15% Precast gel of TL2 induction, (COOMASSIE w/alk. cell  
 Sol/in sol fractions Western Blot 20  $\mu$ /lane each Sample

1: 2500 dilution TL2 antisera (CK9B Ab43)  
 1: 5000 dilution anti-Robust



gel is overloaded  
 and for Antisera is  
 not diluted enough.

Some encouraging results  
 (1) Antisera recognizes antigen

(2) Antisera recognizes  
 short form of TL2  
 in supernatant at  
 28°C and possibly  
 32°C

This Western must be  
 re-run loading less sample and diluting primary antisera more.

Input RABBITS CK9A CK9B WUL DETIDITZ TL2 Long  
 Input Robusts CK9A CK9B WUL CCA2 peptide

13 DO TRANSFECTION of MTALII CCA2 Long #5 into 52 cells

PMALII CCA2 Long #5	750 ng/1	25 $\mu$ l
PC6 reagent	910 ng/1	1.5 $\mu$ l

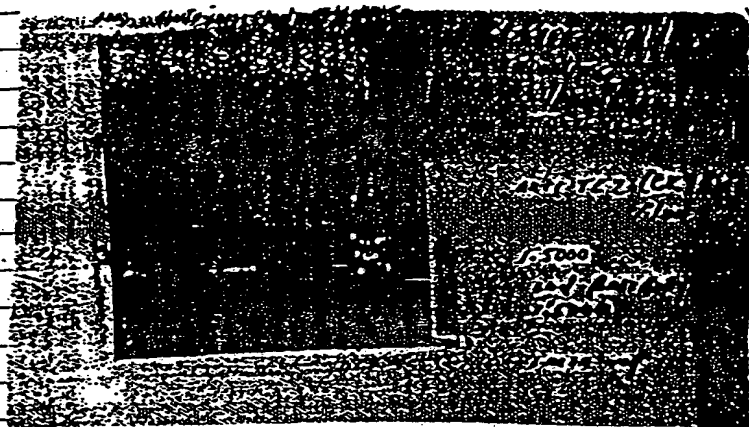
SCIENTIST SIGNATURE

Ed. Huf

DATE 3/5/96

24098-194

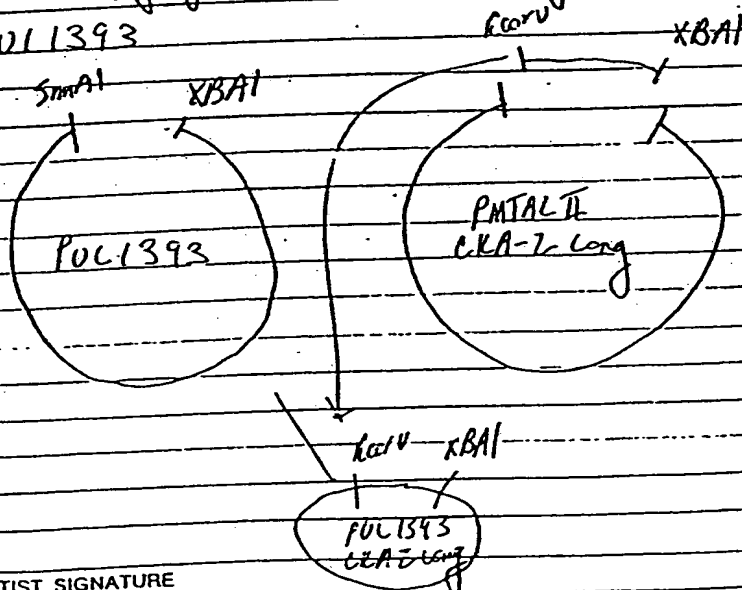
Re-run Western blot of TH2 induction using 4th lane sample and antisera at 1:5000 dilution



15 DNA sequence of PMTALII CKA-2 Long is AS expected

Strategy of CKA-2 Long Cloning is "Bacterial Vector"

POL1393



SCIENTIST SIGNATURE

Kotkuf

DATE 5/5/96

No. 24095-196

yield of URAZ - long K<sub>2</sub>CO<sub>3</sub> (VRA) must enter (10 mg/l)

323

Setup ligation

4 1393

4 1393

6 UAS long

4 ul

PCR 1393 (0.5 mg/l VRA) 4 ul

UAS long (0.5 mg/l VRA) 4 ul

6 ul

7 ul

11 ul

6 ul

Sx. Bcl. I. ligase

6 ul

6 ul

Ligase

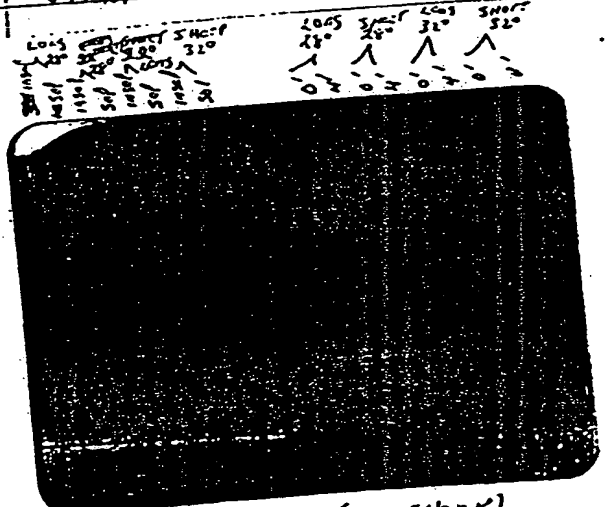
1 ul

1 ul

16°C 8/11

LB 2525 TRANS Form in DH5α

Run agarose gel of 50/1000 fractions GE 698 induction  
of DET1 DET2 TC2 (Pg 191) Also replot Western using Pet 1  
Antiserum

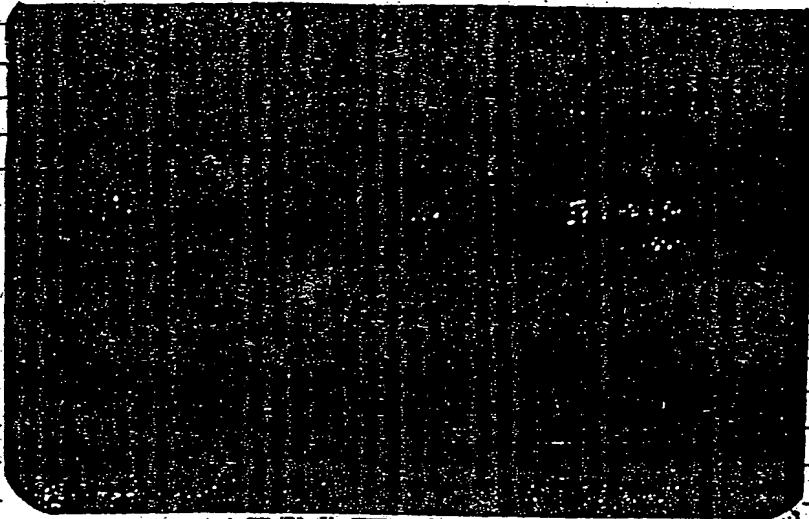


DET1 DET2 TC2 (LONG, 5000)  
GE 698 INDUCTION

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*[Signature]*

DATE 3/5/9



2.6 Coomassie and Western Blot using Anti AAT1 showed solubility of AAT1 AAT2 TC2 slant

Do 1 liter induction of AAT1 AAT2 & TC2 (Long and Slant) for Seibert's Purification

OD<sup>600</sup> of overnights (28°C)  
 TC2 - long - 2.2  
 TC2 Slant 2.15

(28°C) Dilute O/N 1:10 (50 ml / 450 ml induction medium)

OD<sup>600</sup> at time of dilution

OD<sup>600</sup> Addition of Tryptophan Harvest (4 hr)

1 TC2 Slant (500 ml)	.191	.410	2.0
2 " "	.237	.456	1.86
3 TC2 Long	.776	.404	1.82
4 " "	.734	.410	1.84

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*E. J. [Signature]*

DATE 3/5/96

No. 24098-198

3 28

DETI DETZ TLE 49 scale (sec 24)

STRATIGRAPHIC EAGLEVIEW 22 23/23/24 1111212 61198

Long long short short

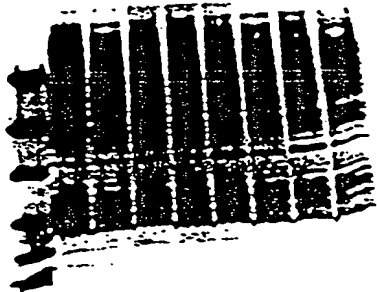
IMAGE SIZE (640 x 480 x 81)

INTEGRATE PERIOD IS 0.01 SECONDS ON 3 COUNTS

IMAGE CREATED ON SUN MAR 23 11:21:29 1996

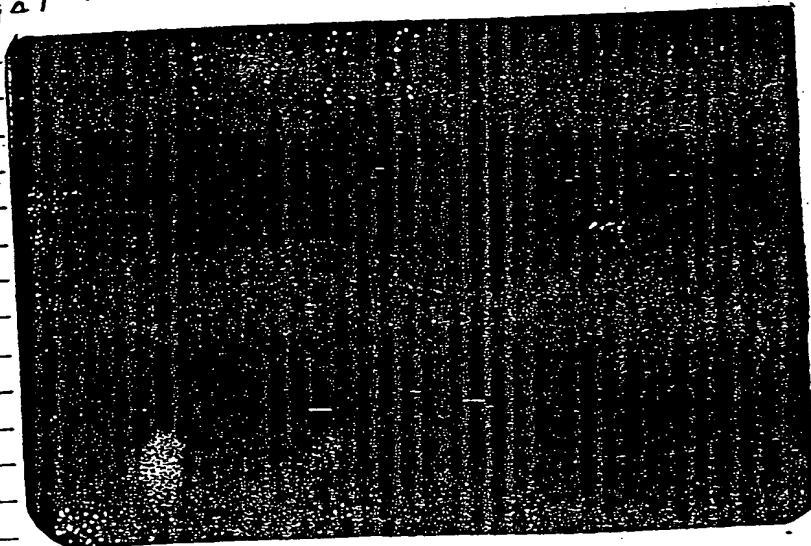
2800

0 4' 0 4' 0 4' 0 4' 0 4' 20 d/ful



Seawater above samples for sal/insal studies

Western Blot - Probe w/ul (Anti DETI - 178.1) and (Anti TLE  
CR501)



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Ad 1 ful

DATE 3/5/96

material (TL2-Skat/STMT looks soluble with DAPI anti-sera  
Turnover to SIX1 FISH)

29 More colonies present on pHL393 CLAR long than Vector  
Please

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Ed. Puf

DATE

3/5/96

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